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13. ABSTRACT *(Maximum 200 Words)*

We have demonstrated that a non-immunogenic Dunning's rat prostate cancer cell line, MATLyLu, had become immunogenic by reducing the endogenous production of TGF-β1. MATLyLu cells are immunogenic, when the endogenous production of TGF-β1 is down regulated. The objective of the project was to evaluate the role of TGF-β in prostate cancer progression. There are three specific aims. Specific aim 1 was to study the effect of TGF-β expression on the in vivo and in vitro growth of the rat prostate cancer MATLyLu cells. Specific aim 2 was to study the effect of TGF-β expression on the host's immune reaction against MATLyLu cells. Specific aim 3 was to assess the effect of TGF-β expression in MATLyLu cells on non-immune hosts. An expression construct containing TGF-β1 antisense was stably transfected into MATLyLu cells. Following transfection, cellular content of TGF-β1 reduced from 70 pg to 10 pg per $2x10^4$ cells and the rate of *in vitro* ^{3}H-thymidine incorporation increased 3-5 fold. After subcutaneous injection of tumor cells into syngeneic male hosts (Copenhagen rats), the tumor incidence was 100% (15/15) for the wild type MATLyLu cells and cells transfected with the control construct, but only 43% (9/21, $p \leq 0.05$) for cells transfected with TGF-β1 antisense. However, when cells were injected into athymic nude rats, the incidence of tumor development was 100% (10/10) for both the wild type MATLyLu cells and cells transfected with the control construct and 90% (9/10) for cells transfected with TGF-β1 antisense. The proposed study is highly relevant to prostate cancer research. In this study, we have demonstrated that TGF-β overexpression by cancer cells can render these cells resistant to host's immune surveillance program. This information is critical for us to propose the Phase II research in TGF-β-based gene therapy in prostate cancer.

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TGF-β, antisense vector, immune response, angiogenesis, extracellular matrix	21
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Table of Contents

Name of the PI: Chung Lee
Title of the Proposal: Expression of transforming growth factor-beta (TGF-β) in prostate cancer progression

INTRODUCTION: Narrative that briefly describes the subject, purpose and scope of the research.
Subject: The present report deals with the role of expression of TGF-β in prostate cancer progression in a rat prostate cancer model, MATLyLu. **Purpose:** The objective of this project is to evaluate the biological role of TGF-β in prostate cancer growth. An aggressive and highly metastatic Dunning's rat prostate cancer cell line, MATLyLu, was used as the model system. Under normal conditions, MATLyLu cells are sensitive to the inhibitory effect of TGF-β. However, like many advanced cancers, MATLyLu cells overproduce TGF-β, which enhances tumor progression in the host. In the present study, we demonstrate that this tumor enhancing effect of TGF-β is mediated through the host immune system. **Scope:** There are three tasks to this project. **In Task 1,** we studied the effect of TGF-β on the in vitro and in vivo growth pattern of MATLyLu cells. **In Task 2,** we have established that the inhibitory effect of anti-sense TGF-β was mediated at least through the activation of host immune system. **In Task 3,** we demonstrated that there also existed a tumor-host interaction that was independent of host immune system.

BODY: Describe the research accomplishments associated with each task outlined in the **approved** Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report.
In Task 1, we studied the effect of TGF-β on the in vitro and in vivo growth pattern of MATLyLu cells.

We observed that under the in vitro conditions, TGF-β exhibited an inhibitory effect against these tumor cells. However, under the in vivo condition, TGF-β enhanced tumor growth. This has been clearly demonstrated that when we downregulated TGF-β expression by using the antisense technology the tumor growth was inhibited.

Briefly, an expression construct containing a DNA sequence in an antisense orientation to TGF-β1 (TGF-β1 antisense) was stably transfected into MATLyLu cells (Isaacs et al, 1981; 1986). Following transfection, cellular content of TGF-β1 reduced significantly. Among 7 positive clones, clones 18 has the best performance. The endogenous production of TGF-β1 in clone 18 has reduced from 70 pg to 10 pg per $2x10^4$ cells and the rate of *in vitro* ^{3}H-thymidine incorporation increased 3-5 fold. Table 1 summarizes the level of TGF-β production and the rate of 3H-thymidine incorporation by the wild type MATLyLu cells, cells transfected with the control vector, and cells transfected with the TGF-β1 antisense vector.

Table 1. Levels of TGF-β1 in cell lysates and ^{3}H-thymidine incorporation rates of wild type MATLyLu cells, cells transfected with the control construct and cells transfected with the TGF-β1 antisense construct.

Cell Type	TGF-β1 (pg/$2x10^4$ cells)	^{3}H-Thymidine Incorporation (cpm/$1x10^4$ cells/well)
Wild Type MATLyLu Cells	70.3 ± 7.7	6,804 ± 434
Cells with Control Vector	67.0 ± 11.8	10,774 ± 700
Cells with Antisense Vector (Clone 18)	9.9 ± 1.2*	30,348 ± 2,866*

All values are expressed as mean ± standard error of the mean.
*The value is significantly different from the other values in the same column ($p < 0.05$).

Linear correlation was conducted to analyze the statistical association between the rate of ^{3}H-thymidine incorporation and the level of TGF-β1 production in 7 clones. The calculated correlation coefficient (r) was 0.769 and was statistically significant ($p < 0.05$). When these cells were injected subcutaneously into male Copenhagen rats (250 g. body weight), tumor incidence reduced to less than 50% of that for wild type MATLyLu (WT) cells and for cells transfected with an empty control vector (CV). Table 2 summarizes the

tumor incidence in Copenhagen rats (syngeneic hosts) from the wild type MATLyLu cells, the control vector cells and the clone 18 cells.

Table 2. Tumor incidence of MATLyLu cells inoculated subcutaneously into syngeneic hosts.

	Trial I	Trial II	Trial III	Overall
Wild type MATLyLu cells	5/5(100%)	5/5(100%)	5/5(100%)	15/15(100%)
TGF-β1 antisense transfected cells	3/5(60%)	4/11(36%)*	2/5(40%)	9/21(43%)*
Control construct transfected cells	5/5(100%)	5/5(100%)	5/5(100%)	15/15(100%)

A total of $2x10^5$ cells were injected s.c.

*The value is significantly different ($p < 0.05$) from other values in the same group by the χ^2-square test (Steele and Torrie, 1960; Bender et al, 1982).

We conducted immunohistochemical staining for blood vessels in tumors derived from the wild type MATLyLu cells, cells transfected with the control vector and cells transfected with the antisense vector. However, there was no significant difference between the tumors of the above three groups of animals.

In Task 2, we have established that the inhibitory effect of anti-sense TGF-β was mediated at least through the activation of host immune system.

This was because that when we injected these anti-sense transfected MATLyLu cells into nude rats, the tumor incidence was increased. We have performed irradiation of MATLyLu cells. The objective is to maintain the irradiated cells viable but unable to proliferate. We used the trypan exclusion assay to assess the cell viability and ^{3}H-thymidine incorporation assay to assess the ability of these cells to proliferate. An irradiation dose titration study was conducted. It was noted that MATLyLu cells required a large dose of radiation in order to render them to stop DNA synthesis yet remained viable. These irradiated cells will be used as the tumor vaccine to test their anti-tumor ability in an *in vivo* study.

Table 3. Tumor incidence of MATLyLu cells inoculated subcutaneously into nude rats.

	Trail I	Trail II	Overall
Wild type MATLyLu cells	5/5 (100%)	5/5 (100%)	10/10 (100%)
TGF-β1 antisense transfected cells	5/5 (100%)	4/5 (100%)	9/10 (90%)
Control construct transfected cells	5/5 (100%)	5/5 (100%)	10/10 (100%)

Xenograft growth of tumor cells in athymic hosts offers an opportunity to assess the behavior of tumor development under immune compromised conditions. These animals are deficient in T cells but their natural killer (NK) cells remain functional. If tumors fail to develop in immune competent hosts but grow in immunodeficient hosts, it is likely that the observed difference in tumor incidence is at least due to a functional T-cell immune system. In the present study, this is the case, as the incidence has been significantly reduced in syngeneic hosts when compared with that in the nude rats. Therefore, a reduction in TGF-β1 production in the tumor cells results in an escape from the T cell mediated immunosuppression.

These results provides a proof of principle that TGF-β plays an important role in host immune system. This observation was the basis of our Phase II project, which will render host immune cells insensitive to TGF-β. These cells are expected to eliminate cancer cells in the host.

In Task 3, we demonstrated that there also existed a tumor-host interaction that was independent of host immune system.

Xenograft growth of tumor cells in athymic hosts offers an opportunity to assess the behavior of tumor development under immune compromised conditions. Since these animals are immunodeficient, any difference in tumor growth between treated group and the control group will be attributed as due to non-immune factors. This was demonstrated by the following Figure. Even though, tumor incidence was not significantly different, the tumor growth was smaller for anti-sense transfected MATLyLu cells than the wide-type MATLyLu cells. Clearly, these effects were unrelated to host immune system. These factors would include, for example, angiogenesis and the production of extracellular matrix.

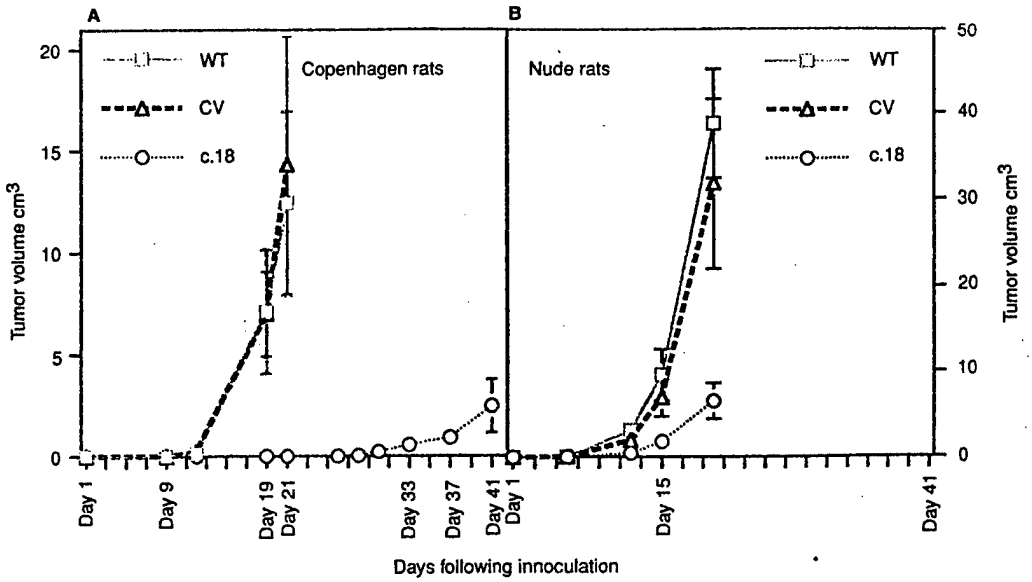

Figure Growth curves of tumours in Copenhagen rats (**A**) (syngeneic hosts) and in nude rats (**B**) (immunodeficient hosts). Tumour volumes were calculated according to the formula described in the text (Janik et al, 1975). Tumour volume in each group was calculated as mean ± SEM of all tumours. Tumours developed from the wild type MATLyLu cells (open squares) and cells transfected with the control vector (open triangles) were significantly bigger with a shorter latent period than those developed from the antisense vectors (open circles). Note that the scales in (**A**) and (**B**) are different, suggesting that tumours grow faster in immunodeficient hosts during the same interval. The vertical bars denote standard error of the mean.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- We have established that rat prostate cancer cells expressing more TGF-β were growth inhibited in tissue culture but their growth was greatly enhanced when they were growing in syngeneic animals.
- We have established that the effect of TGF-β overexpression in tumor cells was at least mediated by an activation of the host immune system.
- We have established that, in addition to the host immune system, TGF-β also facilitated tumor growth through non-immune factors in the host.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research.

1. Manuscripts: We have published two manuscripts as a result of this research. They are:

Matthews E, Yang T, Janulis L, Goodwin S, Kundu SD, Karpus WJ, Lee C. (2000) Down regulation of TGF-β1 production restores immunogenicity in prostate cancer cells. British Journal of Cancer 83:519-525.

Shah AH, Lee C. (2000) TGF-β-based immunotherapy for cancer: Breaching the tumor firewall. Prostate 45:167-172.

2. Abstracts: None
3. Presentations: None
4. Patents: None
5. Licenses: None

CONCLUSIONS: Summarize the results to include the important and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem.

Importance: Many cancer cells, including prostate cancer, are able to over-express TGF-β, leading to aggressive phenotypes (Chang et al, 1993; Steiner et al, 1994; Barrack, 1997). This mutational event is paradoxical, since TGF-β is inhibitory to prostate cancer cells in culture (Morton and Barrack, 1995; Lamm et al, 1997). However, the over-expression of TGF-β alters the host-tumor interaction, which consequently

facilitates tumor growth. The best example is MATLyLu cells. This cell line is similar to late-stage human prostate cancer. MATLyLu is either not immunogenic or weakly immunogenic (Vieweg et al, 1994). The classical studies demonstrated that an over-production of TGF-β in MATLyLu cells was growth inhibitory *in vitro* but growth stimulatory *in vivo* (Steiner and Barrack, 1992; Barrack, 1997). Our current results supported these observations and further concluded that the excessive *in vivo* growth of MATLyLu cells is due to a TGF-β-induced inhibition of the host T-cell mediated immunity (Matthews et al, 2000).

Implications (a novel approach in cancer therapy): TGF-β has been known to be immuno-suppressive. Studies using *in vitro* systems have yielded a complicated and somewhat controversial role of TGF-β in immune regulation (Letterio and Roberts, 1998). The mechanism leading to the activation of these T cells and the acquisition of their effector function (cytokine production) has not been identified. Spontaneous up-regulation of MHC class II and I on multiple tissues was observed to precede lymphocyte infiltration into these organs, suggesting a possible role in TGF-β1 action (Geiser et al, 1993). The use of transgenic mice with an absence of TGF-β signaling specifically in the T cells has led to the autoimmune disease characterized by inflammatory infiltration in several organs (Gorelik and Flavell, 2000). Therefore, TGF-β may regulate T cells homeostasis and prevent inflammatory infiltration. The activation of the T cell immunity in these transgenic studies is consistent with our objective of an increase immune effector function against tumor cells (Shah and Lee, 2000). The proposed Phase II Study takes advantage of the above knowledge and translates into a pre-clinical gene therapy program by using the strategy of the adoptive immuno-therapy. Furthermore, by having the capability of mixing any proportion of the TGF-β insensitive cells and TGF-β sensitive cells of the immune system, the present model system may be able to shed addition light into the role of TGF-β in immune regulation.

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APPENDICES: attach all appendices that contain information that supplements, clarifies or supports that eext.

We attach two journal articles that were published as a result of this research.

Matthews E, Yang T, Janulis L, Goodwin S, Kundu SD, Karpus WJ, Lee C. (2000) Down regulation of TGF-β1 production restores immunogenicity in prostate cancer cells. British Journal of Cancer 83:519-525.

Shah AH, Lee C. (2000) TGF-β-based immunotherapy for cancer: Breaching the tumor firewall. Prostate 45:167-172.

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Down-regulation of TGF-β1 production restores immunogenicity in prostate cancer cells

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Summary The objective of this study is to determine if a non-immunogenic Dunning's rat prostate cancer cell line, MATLyLu, can become immunogenic by reducing the endogenous production of TGF-β1. An expression construct containing a DNA sequence in an antisense orientation to TGF-β1 (TGF-β1 antisense) was stably transfected into MATLyLu cells. Following transfection, cellular content of TGF-β1 reduced from 70 to10 pg per 2×10^4 cells and the rate of in vitro ^{3}H-thymidine incorporation increased 3–5-fold. After subcutaneous injection of tumour cells into syngeneic male hosts (Copenhagen rats), the tumour incidence was 100% (15/15) for the wild type MATLyLu cells and cells transfected with the control construct, but only 43% (9/21, $P \leq 0.05$) for cells transfected with TGF-β1 antisense. However, when cells were injected into immunodeficient hosts (athymic nude rats), the incidence of tumour development was 100% (10/10) for both the wild type MATLyLu cells and cells transfected with the control construct and 90% (9/10) for cells transfected with TGF-β1 antisense. These observations support the concept that MATLyLu cells are immunogenic, when the endogenous production of TGF-β1 is down-regulated.

Keywords: TGF-β expression; rat prostate cancer; immunogenicity; tumour incidence; host–tumour interaction

Although TGF-β is inhibitory to many cancer cells in vitro (Laiho et al, 1990; Pietenpol et al, 1990), most tumours that express large quantities of TGF-β exhibit an aggressive phenotype (Barrack, 1997). On the other hand, tumours that have a reduced production of TGF-β show an attenuated growth pattern in vivo (Fakhrai et al, 1996). This difference of in vitro and in vivo responses of tumour cells to TGF-β is well known. What remains unclear is the impact by the host immune system on in vivo tumour growth due to an overproduction of TGF-β. The present report described the role of TGF-β production in host immune surveillance program against tumour growth.

Among the Dunning's rat prostate tumours, MATLyLu is the most aggressive line (Issacs et al, 1981; 1986). The MATLyLu system has been well characterized and is considered to be a good model for the late-stage, aggressive form of human prostate cancer (Smolev et al, 1977). These cells are androgen-independent and are highly invasive and metastatic. MATLyLu cells are known to be either non-immunogenic, or at best, weakly immunogenic (Shaw et al, 1987; Vieweg et al, 1994). They also produce a large amount of TGF-β1 (Steiner and Barrack, 1992; Barrack, 1997). Since TGF-β is a potent immunosuppressant (Letterio and Roberts, 1998), the lack of immunogenicity in these cells may be due, at least in part, to the large amount of endogenous production of TGF-β. MATLyLu cells are sensitive to TGF-β (Morton and Barrack, 1995). They demonstrate the typical paradoxical in vitro verses in vivo growth pattern in response to TGF-β1 (Barrack, 1997). Under culture conditions, TGF-β inhibits proliferation of MATLyLu cells. However, in animals, TGF-β1 enhances the tumorigenicity. This enhanced tumorigenicity may be the result of tumour–host interaction through a multitude of pathways.

In the present study, we demonstrate that the immunosuppressive effect of TGF-β plays a major role in MATLyLu tumorigenicity. The present results allow us to conclude that MATLyLu cells can be immunogenic when the endogenous production of TGF-β has been suppressed.

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MATERIALS AND METHODS

MATLyLu cells and culture conditions

MATLyLu cells were kindly provided by Dr John Isaacs of Johns Hopkins University at passage 53. Cells were routinely maintained in RPMI 1640 medium (Gibco Life Technologies, Gaithersburg, MD, USA) with 10% FBS (Summit, Ft. Collins, CO, USA), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) (Gibco), and 250 nM dexamethasone. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells under selective pressure were cultured as above with the addition of G418-sulphate (1000 μg ml^{-1}). Transfection of expression constructs to MATLyLu cells was performed at passage 71 and cells were used for the present study between passages 80–90.

Construction of the TGF-β1 antisense expression vector

The pTARGET plasmid (Promega, Madison, WI, USA) is a mammalian expression vector containing the human cytomegalovirus immediate–early promoter region to allow constitutive expression of the cloned vector in host cells. This plasmid also contains a neomycin resistance gene, which was used for selection by G418-sulphate. The vector consisted of base pairs (bp) 433–1461 of the rat TGF-β1 cDNA in the reverse orientation

and was inserted into the multiple cloning site of the pTARGET plasmid. Resulting constructs were digested with restriction enzymes and sequenced to confirm correct orientation, and designated as the TGF-β1 antisense construct. A construct with the empty vector (pTARGET plasmid alone) was prepared in the same manner, and designated as the control construct.

Transfection and cloning

MATLyLu cells were transfected with the TGF-β1 antisense and control constructs with the Lipofectamine™ transfection system (Gibco) according to the manufacturer's recommended procedure. Briefly, cells were suspended in Opti-mem transfection media and were treated with a mixture of Lipofectamine™ and the above constructs (TGF-β1 antisense or control) for 18 h. Following transfection, cells were cultured with the maintenance medium and selected with G418-sulphate. Three days later, these cultures were transfected again and this procedure was repeated for a total of five times. Cloning was performed by limiting dilution into 96-well plates (Costar, Cambridge, MA, USA). Seven days after the initial plating, cells in a single colony were harvested and transferred into 24-well plates. At confluency, these cells were subsequently transferred into 25 cm^2 flasks and further expanded. Prior to injection of these cells into animals, the following tests were conducted.

Verification of cloned MATLyLu cells by PCR

Confluent cultures were trypsinized and DNA was extracted via the Qiagen DNA preparation kit (Qiagen Inc, Chatsworth, CA, USA). DNA contents were measured by UV absorbence. An aliquot of 1.0 μg of genomic DNA was introduced into each PCR reaction vessel along with primers specific for a sequence within the vector. DNA sequences for the respective primers are listed below.

5′ primer-5′-GCACC AAAAT CAACG GGACT-3′ (bp 619–638)

3′ primer-5′-GAGAG AAAGG CAAAG TGGAT GTC-3′ (bp 995–1017)

An aliquot of 10 μl reaction buffer, containing 0.5 μg of Taq polymerase (ISC Bioexpress, Kaysville, UT, USA), 25 mM of dNTPS, 3 mM of $MgCl_2$, was added into each PCR reaction vessel and incubated for 35 cycles in a thermocycler (MJ Research, Watertown, MA, USA). Each cycle consisted of 95°C for 2 min, 55°C for 1 min and 72°C for 1.5 min. The PCR products were subjected to electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and were visualized under a UV lamp. The expected size (399 bp) of the PCR product was determined according to the DNA size reference, which was placed in the first and last lanes in the agarose gel for electrophoresis. Portions of the PCR products were also subjected to restriction digest and, then, to agarose electrophoresis to confirm the expected sequence.

In vitro ^{3}H-thymidine incorporation

^{3}H-thymidine incorporation was carried out to assess proliferative potential of each clone. Cells were seeded at 10 000 cells per well in 96-well plates and cultured in serum-free media. Twenty-four hours later, 1.0 μCi of ^{3}H-thymidine (specific activity, 20–30 Ci $mmol^{-1}$, Amersham, Piscataway, NJ, USA) was added to each well. Cells were incubated for an additional 2 h and were subjected to two freeze–thaw cycles to detach cells from the wells. Suspended cells were transferred to a glass filter (Packard Instrument Company, Meriden, CT, USA) and were lysed with distilled water. Plates were allowed to dry for 2 h and an aliquot of 50 μl scintillation fluid was added. The amount of radioactivity was measured in a Scintillation Top Count Plate Reader (Packard Instrument Company). Results were expressed as counts per minute per well.

Preparation of tumour and cell lysates for TGF-β1 determination

Levels of TGF-β1 in cell lysates and tumour tissues were determined by enzyme-linked immunosorbant assay (ELISA). Tumour tissues, weighing from 200–600 mg, were frozen with liquid nitrogen and ground to a powder. The solubilizing solution was added to each sample at a rate of 3 ml (phosphate buffer, 100 μg leupeptin) per 100 mg of tissue. Cell lysates were prepared by addition of 3 ml of the solubilizing solution to cell pellets (10×10^6 cells). These samples were subjected to constant shaking at room temperature for 4 h and centrifuged for 15 min at 1500 ***g***. The clear supernatants were used for TGF-β1 determination. Briefly, an aliquot of 100 μl of the supernatant was activated with 100 μl 2.5 N HCl and 10 M Urea for 10 min. The activation step was necessary, as the amount of TGF-β1 in untreated samples was always negligible. Samples were neutralized with 100 μl of 2.7 M NaOH and 1.0 M HEPES prior to assay for TGF-β1. Samples were diluted 1:30 in calibrator diluent and subjected to ELISA for TGF-β1 (R&D, Minneapolis, MN, USA) according to the manufacturer's suggested procedure. Results were reported as pg TGF-β1 per 50 μg of tumour tissue or per 2×10^4 cells.

Experimental animals

Copenhagen male rats (syngeneic hosts), Lewis male rats (allogeneic hosts), and athymic nude rats (immunodeficient hosts) were purchased from Harlan Industry (Indianapolis, IN, USA) at 60 days of age. Animals were kept in a temperature controlled room (23 ± 2°C) with tap water and normal rat chow provided ad lib. Experiments started at least 1 week after animals arrived at the facility. They were anaesthetized under methoxyflurane vapour (Schering-Plough, Union, NJ, USA). MATLyLu cells (2×10^5 cells for syngeneic hosts and immunodeficient hosts, 1×10^6 cells for allogeneic hosts) were suspended in 0.2 ml of serum-free culture medium and injected subcutaneously with a 25-guage needle into the left flank near the hind leg, while the animals were under anaesthesia. All procedures were approved by the Institutional Animal Care and Use Committee.

Tumour measurement and tumour histology

Seven days following injection, rats were palpated twice weekly in order to monitor tumour development and tumour progression. When a tumour was palpated, the interval between tumour cell injection and tumour development was considered as the tumour-latent period. Tumour size was measured with calipers and tumour volume was determined by applying the formula $(0.5236 (W + L) (W \times L))/2$, where W represents the width and L is the length (Janik et al, 1975). Animals were euthanized at 21–23 days by

decapitation while under anaesthesia with methoxyflurane vapour. Tumours were dissected and weighed. Tumours were cut into small pieces and were snap-frozen in liquid nitrogen for PCR analysis. A portion of each tumour was fixed in 10% neutral formalin. Tissues for histologic studies were embedded in paraffin, cut at 6.0 μm thick, and stained with haematoxylin and eosin. Photomicrographs of representative sections of the tumour tissues were taken with a camera mounted on a microscope (Olympus Model BH-2) as a hard-copy record.

Statistical analysis

All numerical data were expressed as mean ± standard error of the mean (SEM). All in vitro experiments were repeated at least three times. Data were analysed using the analysis of variance test and Duncan's new multiple range test (Bender et al, 1982; Steele and Torrie, 1960). The Chi-square test was used to determine differences in tumour incidences in animals. A linear correlation was conducted to test the degree of association between the value of ^{3}H-thymidine incorporation and the level of TGF-β1 production for different clones. A correlation coefficient (r) was calculated based on the analysis. A P value of less than 0.05 was considered as statistically significant (Steele and Torrie, 1960; Bender et al, 1982).

RESULTS

Verification of TGF-β1 antisense transfection in MATLyLu cells

A TGF-β1 antisense vector was created by inserting the full-length rat TGF-β1 cDNA into the multiple cloning site of the pTarget vector in the reverse orientation. MATLyLu cells were transfected with this construct, selected with G418-sulphate, and re-transfected in order to assure that high copy numbers of the vector were present. The repeated transfection appeared necessary, as MATLyLu cells produce high levels of TGF-β1. Early attempts using a single transfection episode were unsuccessful in reducing TGF-β1 production in transfected cells. It is likely that a great measure of antisense RNA must be present in the cytoplasm in order to suppress TGF-β1 production. Following serial dilution, clones were chosen. DNA from these cloned cells was isolated for PCR analysis. The positive detection of the expected 399 bp PCR product derived from transfected cell lines confirmed the presence of the transfected construct. Levels of TGF-β1 in cell lysates, as determined by ELISA, were significantly lower in clones transfected with the TGF-β1 antisense vector than in those of wild type MATLyLu cells and cells transfected with the control construct.

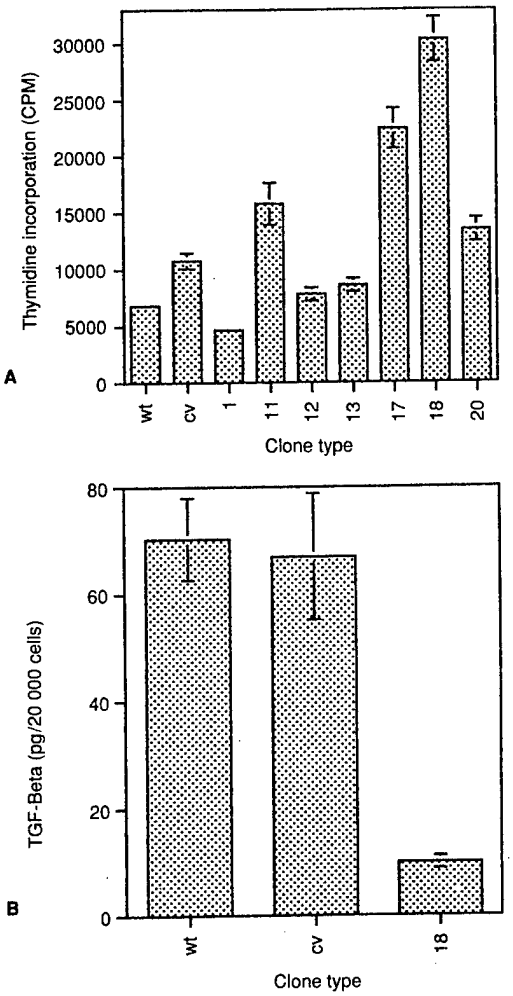

Figure 1 The proliferation rates of wild type cells (WT), cells transfected with the control vector (CV), and different antisense clones in vitro (**A**), ^{3}H-thymidine incorporation, and levels of TGF-β1 production (**B**) ELISA. Clone 18 (c18) was selected because it was the fastest growing and had the lowest level of TGF-β1 production of all of the antisense clones that were screened

The clone with the lowest level of TGF-β1 (clone 18) was chosen for further studies (Table 1 and Figure 1).

^{3}H-Thymidine incorporation assay

^{3}H-thymidine incorporation in these cells was measured to determine proliferation rates of the various positive clones. There was a negative correlation between values of ^{3}H-thymidine incorporation

Table 1 Levels of TGF-β1 in cell lysates and ^{3}H-thymidine incorporation rates of wild type MATLyLu cells, cells transfected with the control construct and cells transfected with the TGF-β1 antisense construct

Cell type	TGF-β1 (pg per 2×10^4 cells)	^{3}H-Thymidine Incorporation (cpm per 1×10^4 cells per well)
Wild type MATLyLu cells	70.3 ± 7.7	6804 ± 434
Cells with control vector	67.0 ± 11.8	10 774 ± 700
Cells with antisense vector (Clone 18)	9.9 ± 1.2[a]	30 348 ± 2 866[a]

All values are expressed as mean ± standard error of the mean. [a]The value is significantly different from the other values in the same column ($P < 0.05$)

Table 2 Tumour incidence of MATLyLu cells inoculated subcutaneously into syngeneic, allogeneic, and immunodeficient hosts

	Trial I	Trial II	Trial III	Overall
Syngeneic hosts:				
Wild type MATLyLu cells	5/5 (100%)	5/5 (100%)	5/5 (100%)	15/15 (100%)
TGF-β1 antisense transfected cells	3/5 (60%)	4/11 (36%)[a]	2/5 (40%)	9/21 (43%)[a]
Control construct transfected cells	5/5 (100%)	5/5 (100%)	5/5 (100%)	15/15 (100%)
Allogeneic hosts:				
Wild type MATLyLu cells	4/4 (100%)	–	–	4/4 (100%)
TGF-β1 antisense transfected cells	0/5 (0%)[a]	–	–	0/5 (0%)[a]
Control construct transfected cells	4/5 (80%)	–	–	4/5 (80%)
Immunodeficient hosts:				
Wild type MATLyLu cells	5/5 (100%)	5/5 (100%)	–	10/10 (100%)
TGF-β1 antisense transfected cells	5/5 (100%)	4/5 (80%)	–	9/10 (90%)
Control construct transfected cells	5/5 (100%)	5/5 (100%)	–	10/10 (100%)

All values are expressed as mean ± standard error of the mean. [a] The value is significantly different ($P < 0.05$) from other values in the same group by the χ^2-square test. In syngeneic hosts and immunodeficient hosts, a total of 2×10^5 cells were injected s.c. In allogeneic hosts, a total of 1×10^6 cells were injected s.c. Three separate trials were conducted for syngeneic hosts. One trial was conducted for the allogeneic hosts and two trials were conducted for immunodeficient hosts.

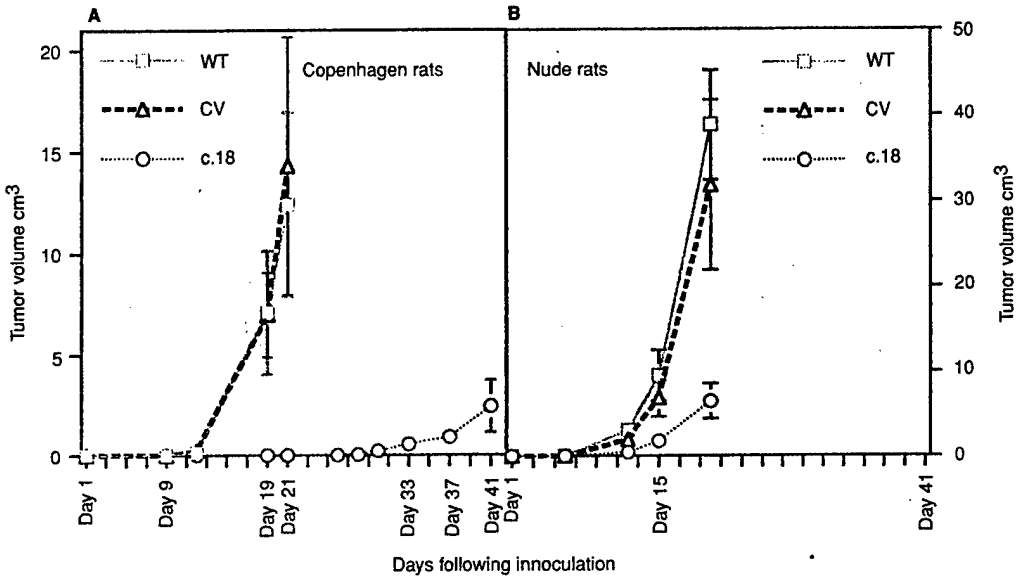

Figure 2 Growth curves of tumours in Copenhagen rats (**A**) (syngeneic hosts) and in nude rats (**B**) (immunodeficient hosts). Tumour volumes were calculated according to the formula described in the text (Janik et al, 1975). Tumour volume in each group was calculated as mean ± SEM of all tumours. Tumours developed from the wild type MATLyLu cells (open squares) and cells transfected with the control vector (open triangles) were significantly bigger with a shorter latent period than those developed from the antisense vectors (open circles). Note that the scales in (**A**) and (**B**) are different, suggesting that tumours grow faster in immunodeficient hosts during the same interval. The vertical bars denote standard error of the mean.

and TGF-β1 levels in different clones ($r = 0.769$, $n = 7$, $P < 0.05$). Clone 18 has the highest level of ^{3}H-thymidine incorporation, which was 3- to 5-fold higher than the proliferation rates for the wild type cells and cells transfected with the control construct (Table 1 and Figure 1). Therefore, this clone was chosen for further investigation.

Tumour-latent period, incidence, and progression of MATLyLu cells in animals

The tumour-latent period is defined as the interval between tumour cell inoculation and detection of palpable tumour nodules. Table 2 shows, in male Copenhagen rats (syngeneic hosts), 100% of tumour development from wild type MATLyLu cells (15/15) and cells transfected with the control construct (15/15); but only 43% in animals injected with clone 18 cells (9/21). The average latent period for tumour development was 12 ± 0.54 days (mean±SEM) for the wild type MATLyLu cells and cells transfected with the control construct, for clone 18 cells, the average latent period was 25.2 ± 3.4 days ($P < 0.05$). Furthermore, tumours derived from clone 18 cells had an average weight (2.5 ± 1.3 g at day 41) significantly less than those derived from wild type MATLyLu cells (15.5 ± 4.3 g) and cells transfected with the control construct (12.6 ± 3.7 g) at day 21 (Figure 2A).

The experiment was repeated in male nude rats (immunodeficient hosts) and in Lewis rats (allogeneic hosts). As indicated in Table 2, tumour incidence of clone 18 cells grown in nude rats was 90% (9/10), which was not significantly different from those of wild type MATLyLu cells (10/10) or of cells transfected with the control construct (10/10). The latent period (8–11 days) of tumour

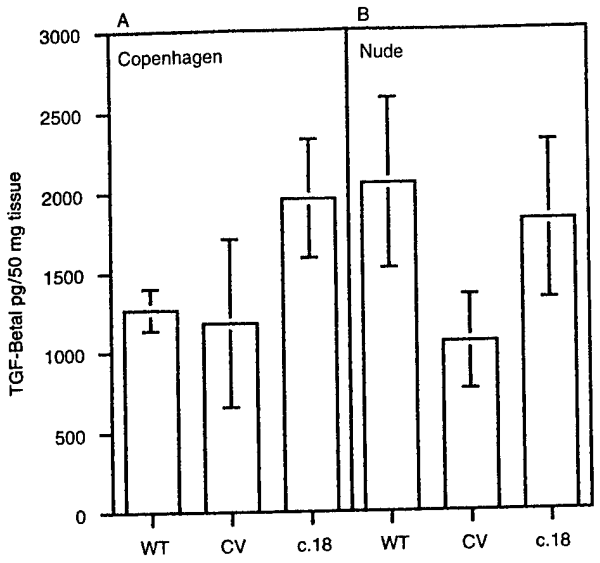

Figure 3 Levels of TGF-β1 in tumours derived from Copenhagen rats (**A**) (syngeneic hosts) and from nude rats (**B**) (immunodeficient hosts). There were no statistical differences in TGF-β1. TGF-β1 levels (pg 50 mg^{-1} tumour tissue) from wild type cells (WT), cells transfected with the control vector (CV), and cells transfected with the antisense vector (c18). Results are expressed as mean ± SEM for at least five tumours in each group. TGF-β1 was determined by ELISA as described in the text. The vertical bars denote standard error of the mean

emergence was not significantly different, in contrast to the extended latent period observed in the syngeneic hosts. Furthermore, the tumour mass of clone 18 cells was significantly smaller ($P < 0.05$) from that of wild type cells or cells transfected with the control construct (Figure 2B). This observation suggests that for clone 18 cells, the host–immune responses as well as non-immune responses contributed to the reduced tumour growth in Copenhagen rats. Tumour incidence in Lewis rats (allogeneic hosts) was also studied in a small series. Results indicated that, in Lewis rats, there was a 100% tumour development for wild-type MATLyLu cells (4/4), 80% for cells transfected with the control construct (4/5) and 0% in animals injected with clone 18 cells (0/5) (Table 2).

Characterization of MATLyLu tumours

Histologic features of tumours were similar to those reported in the literature (Isaacs et al, 1981, 1986). They contained highly undifferentiated tumour cells, resembling those of Gleason 5 anaplastic prostate adenocarcinoma. Mitotic figures were frequently seen. When tumour size was greater than 1.0 cm in diameter, central necrosis was evident. The periphery of the tumour was always surrounded by fibrous tissue derived from the host. In the present study, since tumours were harvested at a relatively early stage, the incidence of metastasis was not frequent. There were no apparent differences in morphologic features of tumours harvested from different treatment groups.

Results of PCR analysis for the presence of the expression vector indicated that clone 18 cells contained the expected 399 bp PCR product. Levels of TGF-β1 in the tumour lysates were determined by ELISA. As shown in Figure 3, in syngeneic as well as in athymic rats, tumours derived from the three different cell types showed comparable levels of TGF-β1. These results suggest that tumours derived from these cells all contain relatively high levels of TGF-β1 regardless of whether or not the original inoculum produced a low level of the growth factor.

DISCUSSION

Results of the present study have demonstrated that MATLyLu cells, when the production of TGF-β1 is reduced, proliferate more rapidly under in vitro conditions but are less tumorigenic under in vivo conditions. The difference in growth behaviour of tumour cells under these two conditions is likely due to tumour–host interactions. It is widely accepted that wild type MATLyLu cells are non-immunogenic or, at best, weakly immunogenic (Shaw et al, 1987; Vieweg et al, 1994). Many experimental immunotherapy protocols have failed to cure the MATLyLu tumour (Vieweg et al, 1994). Results of the present study have demonstrated that the wild type MATLyLu cells produce high levels of TGF-β1 and are immuno-suppressive (Steiner and Barrack, 1992; Barrack, 1997). By simply reducing the production of the endogenous TGF-β1, MATLyLu cells showed a reduced tumour incidence in immune-competent hosts but not in immune-deficient hosts.

TGF-β is a potent immunosuppressant (Karpus et al, 1991; Leterio and Roberts, 1998). The notion that many tumour cells produce large quantities of TGF-β has been acknowledged (Gomella et al, 1992). Therefore, it is reasonable to propose that many tumour cells are immunosuppressive and that they are able to escape the host immune surveillance system. Basically, the present study observed the effect of TGF-β1 production by the tumour cells on three tumorigenic events: the incidence of tumour formation, the latent period, and the progression of established tumours. The first two events are clearly T-cell mediated, as there are significant differences in tumour incidence and the latent period between the syngeneic hosts and immune-deficient hosts. The third event may involve factors other than T-cell mediated immune system, as the impact of a reduced production of TGF-β1 on tumour progression is apparent in both syngeneic hosts and in immune-deficient hosts. These issues will be addressed below.

Xenograft growth of tumour cells in athymic hosts offers an opportunity to assess the behavior of tumour development under immune-compromised conditions. These animals are deficient in T cells but their natural killer (NK) cells remain functional. If tumours fail to develop in immune-competent hosts but grow in immunodeficient hosts, it is likely that the observed difference in tumour incidence is at least in past due to a functional T-cell immune system. In the present study, this is the case for tumour incidence and the latent period for tumour development, as the incidence has been significantly reduced and the latent period has been significant delayed in syngeneic hosts when compared with those events in the nude rats. Therefore, a reduction in TGF-β1 production in the tumour cells results in an escape from the T-cell mediated immunosuppression.

It is clear that the tumour–host interaction involves factors other than the T-cell immune system. These non-T cell factor may also play an important role in tumourigenicity. Nude rats, although lacking T cells, retain some immune function, as they still have NK cells, which may perform some tumour-suppressive functions. It is likely that the observed retardation in tumour growth in nude rats receiving clone 18 cells may result from the activation of NK

cells due to the low levels of TGF-β1 produced by these cells. Differences in tumour growth may not completely attributed to the host immune system. It is apparent, from the present result, that the host immune system is one of many host factors that can be influenced by the production of TGF-β1 by tumour cells (Karpus et al, 1991; Leterio and Roberts, 1998).

In addition to immune suppressive function, TGF-β1 production by tumour cells can be responsible for many non-immune host factors, which cannot be ruled out at this stage. These are angiogenesis, stromal–epithelial interaction, expression of adhesion molecules, and production of extracellular matrix proteins (Battegay et al, 1990; Welch et al, 1990; Yang and Moses, 1990; Karpus et al, 1991; Barrack, 1997). These factors can promote tumour progression. In an environment of reduced TGF-β1 production, the in vivo tumour growth will be hampered. Results from studies with immunodeficient hosts indicate that tumours derived from the wild type MATLyLu cells and cells transfected with the control construct grow faster than those derived from the TGF-β1 antisense transfected cells do. These results indicate that the high levels of TGF-β1 produced by the wild type MATLyLu cells and by cells transfected with the control construct are able to stimulate a greater degree of tumour growth than that of the TGF-β1 antisense transfected MATLyLu cells. These factors may also contribute toward MATLyLu tumour growth.

An interesting finding in this study is that MATLyLu cells, when their TGF-β1 production is reduced, exhibit a low tumour incidence and a prolonged tumour-latent period in syngeneic hosts. When these tumour lysates were subjected to ELISA, however, the average level of TGF-β1 was not significantly different from that of the wild type tumours. Although results of PCR analysis indicate the presence of the antisense expression construct, the possibility could not be ruled out that some of the clone 18 cells might have lost their antisense constructs. We also acknowledge the possibility that a small fraction of the low TGF-β1-producing cells were never successfully transfected with the antisense vector. In either case, the consequence is the emergence of wild type cells in these tumours. The interesting aspect of this observation is that growth of these tumours appeared to be suppressed. A question has been raised of whether or not these TGF-β1-producing clone 18 cells would grow tumours as large as those derived from wild type MATLyLu cells, if they are left in Copenhagen rats for longer periods. We speculate that they would not grow as aggressively as the wild type tumours. This is because, when the low TGF-β1-producing clone 18 cells were injected into syngeneic hosts, they have been immunized and are capable of rejecting wild type tumour cells at least for a few months. This line of rationale is based on the report by Fakhrai et al (1996). These authors used the same method to transfect TGF-β2 antisense vectors into rat glioma cells and observed a similar reduction in tumourigenicity in syngeneic hosts. Subsequently, they re-challenged the wild type glioma cells to hosts who had previously rejected tumours. These re-challenged cells were also rejected, suggesting that, at that stage, host animals have been immunized to reject tumour cells even if they produce high levels of TGF-β.

In the present study, the acquisition of immunogenicity in low TGF-β1 producing MATLyLu cells was further substantiated by a lack of tumour growth in allogeneic hosts. In a preliminary study, tumours derived from wild type MATLyLu cells were eventually rejected by allogeneic hosts (Lewis rats) at 28 days following the initial tumour-cell inoculation. The growth of MATLyLu tumours in Lewis rats is a typical allogeneic transplantation rejection, which is characterized by an acute T-cell mediated reaction (Sherman and Chattopadhyay, 1993). However, during the first 14 days following inoculation of tumour cells, tumours grew from the wild type MATLyLu cells and cells transfected with the control construct, but not for TGF-β1 antisense transfected cells. This observation suggests that the high TGF-β1-producing tumours are endowed with a potent immunosuppressive shield, which protects them from the initial phase of allogeneic rejection.

In conclusion, the present observations have allowed us to conclude that MATLyLu cells, upon down-regulation of TGF-β1 production, become growth stimulated under in vitro conditions and growth inhibited under in vivo conditions. The in vivo growth inhibition is likely due to the presence of tumour–host interaction. The tumour–host interaction includes the host immune system and non-immune responses. Therefore, we propose that MATLyLu cells are actually immunogenic and that this immunogenic property has been masked by the endogenous production of a high level of TGF-β1. The high level of TGF-β1 in MATLyLu cells is a critical factor that renders these cells non-immunogenic. Reducing the endogenous production of TGF-β1 restores the immunogenicity in these cells. Our future studies will focus on cellular and humoral mechanisms that mediate such restoration of immunogenicity in MATLyLu cells. Understanding this mechanism may lead to the development of therapeutic programs based on lowering the level of TGF-β production in tumour cells.

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The Prostate 45:167–172 (2000)

TGF-β-Based Immunotherapy for Cancer: Breaching the Tumor Firewall

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ABSTRACT: Many malignant cells secrete transforming growth factor-β (TGF-β), a potent immunosuppresant, suggesting that TGF-β production may represent a significant tumor escape mechanism from host immunosurveillance. Establishment of a leukocyte subpopulation with disrupted TGF-β signaling in the tumor-bearing host offers a potential means for immunotherapy of cancer. Downregulation of TGF-β secretion in tumor cells results in restoration of immunogenicity in the host, while T-cell insensitivity to TGF-β results in accelerated differentiation and autoimmunity, elements of which may be required in order to combat self-antigen-expressing tumors in a tolerized host. The rationale, approaches, and potential pitfalls of this strategy will be discussed. *Prostate 45:167–172, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **transforming growth factor-β; immunotherapy; tumor immunosurveillance**

INTRODUCTION

In the three decades since Burnet [1] formally introduced the concept of immunological surveillance of tumors, the field of tumor immunology has gained an appreciation for the potential clinical value of manipulating the immune system to fight various cancers, while at the same time eliciting a substantial amount of skepticism over the validity of the concept itself. While the literal interpretation of Burnet's hypothesis has been largely undermined by years of evidence suggesting that nonvirally induced tumor growth is rarely suppressed by normal immune processes, advances in immunological models, particularly in mice, have indicated that there is clearly an interaction between tumors and cells of the immune system, and have suggested that manipulation of these cells may ultimately lead to therapies which eradicate many types of cancer. Concurrently, technological advances in the field of gene therapy have opened avenues of therapeutic development in human disease never before available, and recently these advances have begun to fulfill the promise of efficacy at the clinical level, notably the recent full correction of X-linked severe combined immunodeficiency (SCID) in two patients [2]. Hence, the fields of gene therapy and immunotherapy for cancer have burgeoned significantly in recent years, with the bulk of research focused on two major aspects of tumor immunology: elucidation of antigens recognized by autologous T-cells, and mechanisms of immune evasion by tumors [3]. The latter is the focus of this review, with an eye towards evaluation of a specific escape mechanism employed by many tumors, namely, the secretion of highly immunosuppressive cytokines into the microenvironment of the tumor.

CURRENT APPROACHES IN TUMOR IMMUNOTHERAPY

A significant proportion of the research in the field of tumor immunology currently focuses on the identification of tumor-specific antigens and the cytolytic T-cells specific for these peptides [4], which fall broadly into one of six classes: shared tumor-specific antigens (MAGE, GAGE), mutated antigens (bcr-abl, CDK4, β catenin), differentiation antigens (melan-A,

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tyrosinase, PSA), overexpressed normal antigens (p53, HER-2/neu), or virally induced antigens (HPV E7). When such peptides are defined, therapeutic approaches have included coadministration of the peptide with adjuvant [5], pulsing peptides onto dendritic cells [6], and subcutaneous injection of naked DNA-expressing tumor antigen [7]. In cases in which the antigen for a particular tumor has not been identified, isolation of tumor-infiltrating lymphocytes (TILs) and lymphokine-activated killer cells (LAKs) from primary tumors followed by ex vivo expansion with IL-2 and adoptive transfer into the patient has met with various degrees of success, with up to 30% of patients responding to treatment for light tumor burdens [8]. General immunostimulatory approaches using cytokines include IL-2 administration [9,10] as well as modification of autologous tumor cells with interleukin-12 [11], granulocyte-macrophage colony-stimulating factor (GM-CSF) [12], and interferon-γ [13,14]. Modification of costimulatory molecules includes upregulation of B7-CD28 interactions [15,16] between antigen-presenting cells (APCs) and T-cells, as well as downregulation of the immunosuppressive surface molecule cytolytic T lymphocyte antigen 4 (CTLA-4) [17].

Unfortunately, the efficacy of many promising murine models of tumor regression when translated into the clinic for human trials has typically been disappointing; rarely have immunotherapy protocols offered a viable alternative to the treatment of local tumors by conventional means, and even less so in the more pressing problem of metastatic disease. The finding that tumor-bearing mice retain antigen-specific immunity for those same tumor cells [18] presents a troubling caveat for tumor immunotherapy: generation of a specific cytotoxic T-cell response to tumor antigen is unlikely to be sufficient in treating well-established tumors. Thus, the growth of cancer cells in immunocompetent hosts clearly appears to be an active process on the part of the tumor, and not simply a passive lapse on the part of the immune system [19]. Cancer immunotherapies, therefore, will likely have to account for not only the active priming of immunity, but for the countermeasures taken by the tumor itself.

TUMOR ESCAPE FROM IMMUNOSURVEILLANCE

Mechanisms by which tumors effectively evade triggering immune responses in competent hosts include interference with antigen processing and presentation, antigenic variation, lack of costimulatory signals to T cells, induction of apoptosis, and secretion of immunosuppressive cytokines and other soluble factors. Transport-associated peptide (TAP), a critical component of antigen presentation in the class I pathway, is downregulated in some tumor types [20], as is the MHC I complex itself [21,22]. Antigenic peptides expressed on the surface of tumor cells may be downregulated in some tumor types, and this selection phenomenon is of particular concern to therapies directed against a single tumor-specific peptide, which may serve as an in vivo selection mechanism for cells with downregulated expression of the target antigen. Lack of B7 costimulatory molecules on the surface of the tumor cell may induce anergy on the part of the T cells [23,24], while Fas ligand (FasL) expression by the tumor cell may trigger lymphocytic cell death [25]. But by far the broadest strategy of immune evasion, employed by most tumors to varying degrees, is the secretion of such factors as transforming growth factor-β (TGF-β), interleukin-10, and prostaglandin E_2 into the tumor microenvironment, which modulate the immune response in a downregulatory fashion [26]. The existence of antigenic peptides on the surface of tumors despite the lack of an immune response against the tumor as a whole, as discussed above, is strongly suggestive of a mechanism by which tumor cells actively downregulate the tumoricidal capabilities of antigen-specific T-cells which localize to and, in some cases, infiltrate the tumors themselves. This last line of defense on the part of the tumor, which we here characterize as the tumor "firewall," may help to explain the significant amounts of data in the immunotherapy field which indicate that specific, activated T-cells in tumor-challenged hosts exist and may even undergo clonal expansion, but do not result in ablation of the tumor. Hence, it appears that current immunotherapies directed at stimulation of the immune system against cancer cells have largely succeeded with regard to a basal level of immune stimulation, yet a final piece of the puzzle remains unresolved, and that is a methodology by which the cytokine-mediated immunosuppressive defense mechanism of the tumor cells can be breached [27]. We propose that modulation of the host immune system so as to produce leukocytes insensitive to the most potent of the secreted immunosuppressants, TGF-β [28], may provide a means to circumvent the tumor firewall and effect a total remission of the tumor, particularly in conjunction with therapies which promote an inflammatory response in the local tumor microenvironment. The remainder of this review will focus on the potential uses and pitfalls of TGF-β-mediated immunotherapy for cancer.

TGF-β OVERVIEW

TGF-β represents a family of pleiotropic, secreted growth factors which regulate such diverse processes as embryonic development, wound healing, organ de-

velopment, and immunoregulation [29]. Of the three known mammalian isoforms, TGF-β1 represents the most well-defined member of the family, and accounts for the large majority of the literature to date. A 25-kDa disulfide-bonded homodimeric peptide, TGF-β must be proteolytically cleaved into its active form by multiple possible pathways [30], though definitive mechanisms for in vivo activation of the latent precursor remain somewhat elusive [31].

Signal transduction of TGF-β ligands is mediated primarily through TGF-β type I and type II receptors, which are expressed on a wide variety of cell types, usually at low levels [32]. The current paradigm for signaling through these surface receptors involves recruitment of type I receptors by ligand-bound type II receptors, forming a tetrameric complex which transduces the signal through an intrinsic serine-threonine kinase activity [33]. With regard to TGF-β1, it is important to note that only the type II TGF-β receptor is capable of binding ligand, whereas the type I receptors are in contact with the ligand but do not have direct ligand-binding capability [34]. Heteromeric interaction between type I and type II TGF-β receptors requires functional kinase activity in the type II receptor cytoplasmic tail, thus making the cytoplasmic domain critical for recruitment of the type I receptor into the complex upon ligand binding. The implication of this observation is that TGF-β1 signaling in a type II TGF-β receptor with a mutated cytoplasmic domain is abrogated despite the presence of the functional type I receptor, making the type II receptor an ideal target for gene therapy protocols aimed at modulating the TGF-β1 signaling pathway (see below).

ROLE OF TGF-β IN THE TUMOR FIREWALL

A wealth of evidence in murine tumor models indicates that a cytokine firewall exists, and that the principal component of this defense is TGF-β [35]. Modification of highly immunogenic C3H tumors with a TGF-β expression vector allowed for growth and escape from immunosurveillance in vivo, despite a lack of downregulation of MHC I or tumor-specific antigen [36]. A TGF-β-targeted vaccine approach in rat gliomas has been reported to result in the complete eradication of 100% of tumors when an antisense TGF-β construct was introduced into resected tumor cells ex vivo and then locally reintroduced into the tumor-bearing host [37]. In a mouse thymoma model, tumor cells engineered to secrete a soluble TGF-β type II receptor resulted in a suppression of tumorigenicity [38], again strongly suggesting that interference with the cytokine profile of the tumor microenvironment may make it vulnerable to normal immune surveillance processes. One could reasonably propose that such an approach would prove additive in combination with immunostimulatory strategies now in development, particularly IFNγ and GM-CSF-based therapies. Our own work has indicated that the TGF-β firewall is also a potent factor in urologic tumor models, notably the Dunning rat prostate tumor MATLyLu. Stable transfection of MATLyLu cells with a TGF-β antisense vector causes conferral of immunogenicity in Copenhagen rats, resulting in the inability of these modified cells to form a tumor in immunocompetent hosts, though they remain tumorigenic in nude rats [39].

The modification of tumor cells in a vaccine approach may ultimately be limited, however, in the eradication of well-established tumors in humans, as tumors with particularly strong immunosuppressive firewalls may prove invulnerable to vaccine-primed antigen-specific lymphocytes. The effect of TGF-β in this mode may be the result of a direct effect on both the proliferation and effector functions of the cytolytic cells themselves [40–42], or by biasing the APC preference towards activation of T-cells bearing an antiinflammatory Th2 phenotype [43]. In any case, the vaccine approach may prove to be effective as a sort of specific adjuvant, but it is likely necessary for maximum efficacy that the immune system itself, and not simply the tumors, should benefit from engineering directed at allowing leukocytes to overcome TGF-β-mediated immunosuppresion. Such a gene therapy-based methodology will seek to establish a population of leukocytes in the host which are TGF-β insensitive, and which therefore will be capable of activation and localization to the site of both primary tumors and metastases, and will also not have their tumoricidal properties downregulated upon arrival at those sites.

A GENE THERAPY APPROACH TO OVERCOMING TGF-β-MEDIATED IMMUNOSUPPRESSION

Clinical data from human gene therapy trials in the past decade have indicated that therapeutic regimens directed at modification of a patient's immune cells, particularly T-lymphoctyes, may be more efficiently addressed by targeting hematopoietic stem cells (HSCs) in the bone marrow rather than mature effector cells in the periphery. Compensations for T-cell defects, such as the aforementioned X-SCID, have been shown successfully to benefit by viral transduction of autologous stem cells with the corrective gene. Recent improvements in gene transfer technology, such as the use of Flt-3 ligand in human HSC culture and infection on fibronectin-coated plates, have dramatically increased the probability of success of such an approach directed at HSCs, and this trend is likely

to continue with the development of safer lentivirus-based retroviral vectors which are capable of integrating transgenes into the chromosomes of nondividing cells [44].

Disruption of the TGF-β-mediated signaling pathway has been efficiently achieved through the use of a dominant-negative TGF-β type II receptor in a transgenic mouse model [45], and represents a viable approach in a gene therapy context. Such a regimen involves isolation of autologous HSCs from the patient, transduction with the virally encoded TGF-β dominant-negative receptor, and reinfusion of the stem cells into the patient. Theoretically, these cells would mature into TGF-β-insensitive cells of both lymphoid and myeloid precursor lineages, offering both effector and APC function at the tumor sites, and would represent a subpopulation of circulating immune cells in the patient which would be demonstrably more effective at breaching the tumor-derived cytokine firewall in both primary and metastatic sites, offering antitumor cytolytic activity that cannot be achieved through tumor modification alone, particularly with regards to established and disseminated cancers. The combination of this approach with an antigen-specific priming strategy, such as peptide pulsing onto dendritic cells or GM-CSF/IFNγ-based engineering of tumor cells, would provide a means of overwhelming the tumor on multiple fronts, both in stimulating the offensive capabilities of the immune cells and in diminishing the efficacy of the tumor's most potent defensive mechanism.

THE GENERATION OF HOST AUTOIMMUNITY

The lack of clinical success of many cancer immunotherapies to date appears to largely be the result of a maintenance of tolerance for the tumor by the host immune system; a TGF-β-mediated approach would, in essence, deliberately provoke an autoimmune response in the patient. TGF-β1 knockout mice display a broad, multifocal inflammatory response and typically die within 3 weeks postpartum [46,47]. This phenotype is somewhat milder in the T-cell transgenic model, in which manifestations of autoimmunity typically take place at age 3–4 months in C57BL/6 mice, which may reflect the presence of active TGF-β modulation of other immune cells, particularly APCs. The induction of a potent autoimmune response in cancer patients, while suggesting a means for tumor eradication [48], also raises significant issues of safety and necessitates that the process be either self-limiting or designed such that the effect is engineered with a manipulatable cutoff switch. Vector technologies such as inducible expression or "suicide gene" constructs may offer a viable means of limiting the duration of the response, or, as a failsafe mechanism, radiotherapy of patients followed by reinfusion of autologous, FACS-sorted HSCs depleted of the transgene-positive population would eliminate the risk of a broader autoimmune phenotype developing. In patients with heavy tumor burdens and widespread metastatic disease, such a dramatic approach may prove necessary and effective in combating the cancer while at the same time representing a substantial improvement in the efficacy-toxicity ratio over both conventional chemotherapeutics and systemic cytokine administration protocols. Evaluation of the TGF-β-directed gene therapy approach in animal models is currently underway in our laboratory, which we hope will establish guidelines for manipulating this potent immunoregulation pathway in vivo so as to maximize the therapeutic benefit of induced autoimmunity, while at the same time minimizing the pathologic consequences of attacking the tumor firewall. Ultimately, a synergistic approach between this protocol and others directed at stimulation of antigen-specific T-cells may prove to be the most beneficial in a clinical setting, and represents a significant direction in the future of cancer immunotherapy.

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Dr. Gerald P. Murphy dedicated his life to prostate cancer research. His dendritic cell-based immunotherapy represents a major breakthrough in the treatment of prostate cancer patients. Consistent with his initiative, we have postulated a novel gene therapy strategy for cancer, which we offer in the context of this issue dedicated to Dr. Murphy.

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